Detection of anticardiolipin antibodies in culture supernatants

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Abstract

Objective
We have attempted to devise a method for measuring the levels of anticardiolipin antibodies produced in in vitro culture of peripheral blood mononuclear cells isolated from patients with systemic lupus erythematosus (SLE), with or without circulating anticardiolipin antibodies, patients with the primary antiphospholipid syndrome (PAPS) and normal controls.

Methods
Peripheral blood mononuclear cells were isolated and cultured for up to six days, in the presence or absence of added cytokines in the culture medium. Supernatants harvested after culture were tested by ELISA for the presence of anticardiolipin antibodies.

Results and Conclusion
Despite variation of the culture conditions and of the cell numbers and populations used, it was found that accurately measurable levels of anticardiolipin antibodies could not be detected reliably in any of the culture supernatants. It is concluded that alternative methods of measurement of antibody production need to be explored.

Key words
SLE, PAPS, anticardiolipin antibodies, in vitro.

Introduction
Anticardiolipin antibodies are detectable in the sera of approximately 30% of patients with systemic lupus erythematosus (SLE) and also in two to three times as many with the primary antiphospholipid syndrome (APS) (1). Patients with the antiphospholipid syndrome (APS) are more likely to have thrombotic events (both arterial and venous) and multiple miscarriages than normal individuals. They may also develop thrombocytopenia and livedo reticularis.

B cells in patients with SLE are in a chronic state of activation and many antibodies are produced spontaneously. These include both autoantibodies and antibodies directed against other exogenous antigens. The cytokine network regulates interactions between and within populations of cells and the cytokine profile has been shown by many researchers to be abnormal in patients with SLE. It is possible to influence the antibody-producing capacity of B cells in vitro through the addition of exogenous cytokines and antibodies to cytokines. A method has been devised in this Department for assessment in vitro of the effect of various cytokines and antibodies to cytokines. The aim of this work was to set up a similar system whereby the ability of B cells to produce anticardiolipin antibodies in vitro could be measured in the same way.

Patients and methods
In total, 25 patients with circulating anticardiolipin antibodies, 5 patients with the primary antiphospholipid syndrome, 3 disease controls (patients with SLE who did not have anticardiolipin antibodies) and 4 normal controls were used in this study. Patients were all attending the SLE clinic at the Middlesex Hospital and were selected on the basis of a history of high levels of serum anticardiolipin antibody as measured by the hospital's routine haematology service. The study had the approval of the hospital's ethics committee. All of the SLE patients met 4 or more of the revised classification criteria for SLE proposed by the American College of Rheumatology (2). The patients with antiphospholipid syndrome (both primary and in conjunction with SLE) were diagnosed according to the Harris criteria (3).

Of those patients studied, all of the PAPS patients were female, between the ages of 23 and 50 years (mean 35.6 years S.D. 13.39). Twenty-three of the 25 SLE patients were female, between the ages of 25 and 70 years (mean 44.3 years S.D. 11.73). The 2 male patients with SLE were aged 31 and 47 (mean 39, S.D. 11.31). The 3 disease controls were all female, aged 25, 40 and 48 years (mean 37.6, S.D. 15.37).

Cell culture
Peripheral blood mononuclear cells (PBMC) were separated from whole heparinised blood by Ficoll gradient centrifugation (Histopaque, Sigma H8889) and washed 3 times in serum-free medium. Viable cells (counted using acridine orange and UV microscope) were then adjusted to the appropriate concentration in RPMI 1640 supplemented with antibiotics and 10% foetal calf serum. The variations in experimental protocol used during this study are summarised in Table I. The initial methodology was in accordance with Salaman (personal communication) and (4). Supernatants from replicate cultures were pooled prior to ELISA testing for the presence of antibody. Samples were tested for the presence of total IgG and IgM and anticardiolipin antibody. Pooled samples yielded a maximum of 1.5 ml supernatant. Patients were frequently lympho paenic, which led to fewer replicates of each culture and a smaller volume of supernatant.

Anticardiolipin antibody assay
Plates were pre-coated with cardiolipin (Sigma C1649), using the research method in general use within this Department to detect anticardiolipin antibodies in the sera of patients with PAPS or SLE and to screen supernatants from cloned cells for the production of monoclonal anticardiolipin antibody. This
Table I. Summary of the variations in the basic cell culture and antibody visualisation techniques used in attempts to detect anticardiolipin antibodies in culture supernatants.

<table>
<thead>
<tr>
<th>Source of cells</th>
<th>Cell culture conditions</th>
<th>Other variations in methodology</th>
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<tbody>
<tr>
<td>1. Standard methodology</td>
<td>PBMC 6 days culture 10^6 cells/ml 0.5 ml culture vol. 10% foetal calf serum</td>
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<tr>
<td>B cells circulate in the blood in low numbers. A higher concentration of cells per ml should increase the likelihood of autoantibody-producing cells being present in culture.</td>
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<td>2. Increased cell numbers</td>
<td>PBMC 6 days culture 2 x 10^6 cells/ml 0.5 ml culture vol. 10% foetal calf serum</td>
<td>IL-6, IL-10 or IL-12 added to cultures to test whether the addition of cytokine could affect total antibody levels or raise ACL production so that it could be detected.</td>
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<tr>
<td>3. Addition of exogenous cytokine</td>
<td>PBMC 6 days culture 2 x 10^6 cells/ml 0.5 ml culture vol. 10% foetal calf serum</td>
<td>IL-6, IL-10 or IL-12 (Sigma 1395, 3519 or 1270)</td>
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<tr>
<td>Antiphospholipid antibodies recognise a complex of anionic phospholipid in combination with 2-GPI present in foetal calf serum (FCS). Therefore, the presence of low levels of ACL antibody may be undetectable in the presence of FCS (6). The absence of serum from the culture would not affect the ELISA assay itself because the FCS used in blocking would complex to cardiolipin bound to the solid phase and thus bind any ACL antibody present.</td>
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<tr>
<td>4. Use of serum-free culture medium</td>
<td>PBMC 6 days culture 2 x 10^6 cells/ml 0.5 ml culture vol Serum-free medium (Sigma Q3128)</td>
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<td>Biotin-labelled anti-human IgG/M was substituted for the alkaline phosphatase conjugated antibody and was followed by streptavidin alkaline phosphatase polymer before development of the colour. Each biotin molecule binds 4 streptavidin molecules and thus will enhance the optical density of the final colour (Sigma B3773, B1265 and S5795).</td>
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<tr>
<td>5. Use of serum-free culture medium</td>
<td>PBMC 6 days culture 4 x 10^6 cells/ml 0.5 ml culture vol Serum-free culture medium</td>
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<td>Lipopolysaccharide (LPS) (Sigma) was used as a B activator at a final concentration of 1 g/ml. LPS was selected in preference to PHA in an attempt to avoid activation of T cells in the culture. Non-specific activation of T cells would result in the release of cytokines and make the interpretation of any data obtained even more difficult.</td>
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<tr>
<td>6. Increased sensitivity of ELISA</td>
<td>PBMC 6 days culture 2 x 10^6 cells/ml 0.5 ml culture vol Serum-free culture medium ELISA dev’d using biotin-labelled anti-human IgG/M and streptavidin alkaline phosphatase</td>
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<td>Efforts to detect spontaneous secretion of ACL antibody being unsuccessful, specific stimulation was attempted with cardiolipin in alcoholic solution being allowed to bind to culture tubes (50 g/ml) prior to cell culture. It was expected that culture in the presence of foetal calf serum could produce detectable levels of ACL antibody, this providing a source of 2-GPI which would enable binding through complexing to cardiolipin bound to the solid phase.</td>
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<td>7. Specific stimulation of ACL production</td>
<td>PBMC 4, 6, or 10 day culture 2 x 10^6 cells/ml 0.5 ml culture vol Serum-free culture medium</td>
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<tr>
<td>Lipopolysaccharide (LPS) (Sigma) was used as a B activator at a final concentration of 1 g/ml. LPS was selected in preference to PHA in an attempt to avoid activation of T cells in the culture. Non-specific activation of T cells would result in the release of cytokines and make the interpretation of any data obtained even more difficult.</td>
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<td>8. Stimulation of all B cells</td>
<td>PBMC 4, 6, or 10 day culture 2 x 10^6 cells/ml 0.5 ml culture vol 10% foetal calf serum</td>
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<td>It is possible that supernatants were being harvested too late in the culture period. The initial methodology followed a system devised by Bourne et al. (5) which did not measure antiphospholipid antibodies in supernatants. Throughout the culture period it is possible that debris released from apoptotic cells, including exposed phospholipid molecules (7), could bind to ACL antibodies so that they would not be available for assay. [It is known that monoclonal ACL antibodies cross-react with other negatively charged phospholipids in ELISA assays (8)].</td>
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<td>9. Time course</td>
<td>PBMC 1.2, 3 or 4 day culture 2 x 10^6 cells/ml 0.5 ml culture vol 10% foetal calf serum ELISA incubated at 4°C</td>
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<td>Binding of ACL to CL on solid phase may be inhibited by incubation at 37°C.</td>
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<td>10. Incubation at 4°C</td>
<td>PBMC 6 days culture 2 x 10^6 cells/ml 0.5 ml culture vol 10% foetal calf serum CL coated onto a plate at a concentration of 40 g/ml</td>
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<td>Concentration of CL on solid phase may be too low for optimum sensitivity.</td>
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<tr>
<td>11. Increased concentration of CL bound to solid phase</td>
<td>PBMC 6 days culture 2 x 10^6 cells/ml 0.5 ml culture vol 10% foetal calf serum CL coated onto a plate at a concentration of 40 g/ml</td>
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<td>Few plasma cells circulate in the blood and it is more likely that cells producing ACL antibodies will be found in large numbers in the spleen. Frozen spleen cells, preserved after splenectomy, were thawed and cultured under the same conditions as PBMCs.</td>
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<td>12. B cells not in the peripheral circulation</td>
<td>Spleen cells from frozen sample 5 days culture 2 x 10^6 cells/ml 0.5 ml culture vol 10% foetal calf serum</td>
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| 253
assay had been developed in the past and the protocol optimised by Ravirajan (personal communication). The assay is sensitive down to 100 ng/ml anticardiolipin antibody. Briefly, cardiolipin in alcoholic solution at a concentration of 20 µg/ml was used at 50 µl per well in round-bottomed ELISA plates (NUNC Maxisorp 4-42404). The solution was allowed to evaporate overnight at 4°C leaving cardiolipin bound to the solid phase. This concentration of cardiolipin was lower than that used by some other investigators, but it had been shown in this Department (Radway-Bright, personal communication) that even in the presence of very low levels of anticardiolipin antibody, such as may be detected in supernatants following fusion and cloning, the sensitivity of the assay was not improved by increasing the concentration of cardiolipin. Cell culture supernatants were added to blocked wells (10% FCS in bicarbonate buffer pH 9.6, 2 hours, 37°C) in 50 µl aliquots in triplicate, both neat and 1:2. Negative controls were blocked uncoated wells. Serial doubling dilutions of serum or plasma both from patients known to have high levels of circulating anticardiolipin antibody and from the individuals being tested were used as positive controls.

**ELISA assay for total IgG/M**

Plates were coated with 50 µl per well of a 5 µg/ml solution of anti-human IgG or IgM (Sigma 15885/16385) in bicarbonate buffer and treated as already described. The assay for spontaneous production of total IgG/IgM into the supernatant was always carried out in parallel with the anticardiolipin assay. This assay served as a positive control for the cultured cells, showing that they had survived the culture period and were capable of producing antibody. In addition, on one occasion cells were counted using acridine orange and UV microscope to visualise live cells at the end of the culture period. Live cells were found still to be present, though in lower numbers than before culture. Plates were developed with alkaline phosphatase conjugated anti-human ACL antibodies in culture supernatants / G.S. Dean & D.A. Isenberg

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**Fig. 1.** (a) The effect of culture volume and the dilution of supernatant on the detectability of total IgG in culture supernatants taken from 6-day cultures of PBMCs from a patient with SLE and the phospholipid syndrome. (b) Control dilution curve showing serial dilutions of a plasma sample taken from a patient with SLE and the phospholipid syndrome. This sample patient can be seen to have high levels of circulating IgG anticardiolipin antibodies.
IgG/M (1 hour, 37°C) (Sigma, A3150, A3275) followed by the alkaline phosphatase substrate (Sigma, N2770) and the colour was allowed to develop for at least 30 minutes prior to reading at 405 nm with a reference filter of 490 nm.

Results
Total IgG and IgM assays yielded significant levels of total antibody present both in the supernatants and in the serum and plasma controls. Figure 1a shows typical examples of the optical densities obtained for total IgG and IgM from supernatant and Figure 1b examples of the control dilution curves. It is evident that the optimum culture volume for future use was 0.5 ml. Dilution of the supernatant caused a sharp decrease in optical density, suggesting that a dilution of no greater than 1:2 could be used in the future. However, the anticardiolipin assays

![Figure 2](https://example.com/figure2.png)

**Fig. 2.** (a) Dilution curves for specimen plasma samples taken from patients with SLE and the phospholipid syndrome, showing high levels of circulating IgG anticardiolipin antibodies using the alkaline phosphatase visualisation system. (b) Dilution curves for specimen plasma samples taken from patients with SLE and the phospholipid syndrome, showing high levels of circulating IgG anticardiolipin antibodies using the biotin-streptavidin system in conjunction with alkaline phosphatase.
were much less satisfactory in outcome. Although the assays were functioning adequately in that readable levels of serum and plasma anticardiolipin antibodies were obtained from the positive control dilution curves (Fig. 2a-b) the levels of anticardiolipin antibodies in the supernatants were apparently too low to be detectable accurately.

The modifications in the experimental protocol used to attempt to improve the sensitivity of the anticardiolipin antibody assay and the rationale underlying each of the changes are summarised in Table I. It was not possible to maximise the probability of detecting antibody by concentrating the supernatants because the culture volumes and numbers of replicates were sufficiently small that too high a proportion of the protein present in the supernatants would stick to the filter and be lost, negating the potential benefit of the concentration step.

It was not found to be possible to detect ACL antibodies in any of the supernatants after culture.

It was also found that the addition of exogenous cytokine did not alter the levels of total IgG/IgM produced in culture. This result, while surprising initially, was in accordance with the findings of Tyrrell-Price (personal communication). An essentially similar system, when used for the detection of anti ds-DNA antibodies spontaneously produced into culture supernatants, showed no difference in the total amount of IgG/M produced spontaneously following the addition of cytokine. However, when the levels of anti ds-DNA antibodies produced were measured, there were detectable levels of antibody present in the supernatant and these levels could be modified by the addition of exogenous cytokine in similar quantities to those used here.

Table II shows sample data of levels of total IgG and IgG anti ds-DNA antibodies seen in culture supernatants from cells derived from 5 patients with SLE and high levels of circulating anti ds-DNA antibodies.

A small study was carried out where blood from 2 patients was assessed for the production of both anti ds-DNA and ACL antibodies in parallel (data not shown). Such parallel studies are generally difficult to accomplish because within the clinical manifestations of SLE, some patients tend to produce high levels of ACL antibodies, while others have high circulating levels of anti ds-DNA antibodies. It was found that while anti ds-DNA antibodies were detectable in samples from both patients, ACL antibodies could not be demonstrated. It was also noted that there was no correlation between the levels of serum antibody detected in the positive controls and whether or not it appeared to be possible to detect any antibody in culture. This finding was not unexpected in that serum antibody levels are generally so much higher than the levels which might be found in supernatant as to make it impossible to compare the two systems. However, the presence of a positive ELISA reading in response to dilute serum served as a positive control for the ELISA system. Use of the biotin:avidin system, a modified visualisation system for the ELISA technique with higher sensitivity enhanced the optical densities measured for control serum samples, but did not improve the readings from supernatants.

Exogenous stimulation of the cells through the addition of cardiolipin bound to the surface of the culture tubes in the presence or absence of foetal calf serum had no effect. It had been expected that the production of detectable levels of ACL antibodies might be seen in the cultures where foetal calf serum was present in the culture medium because 2-GPI present in the serum should be able to form complexes with CL on the surface of the tube and lead to stimulation of specific antibody production. Similarly, when lipopolysaccharide (LPS) was used as a specific B cell activator, no detectable ACL antibodies were found. When spleen cells were taken from 2 patients with SLE after splenectomy, as a way of ensuring the presence of B cells in the cultures, no detectable ACL antibody was found in any of the supernatants.

Modification of the experimental protocol to take into account the possibility that the supernatants were being harvested too late in the culture period showed that while the assay was effective, in that the serum controls showed detectable levels of total IgG, total IgM and ACL IgG/M antibodies and the supernatant samples showed increasing levels of total IgG/IgM from one to four days of culture, once again there was no detectable ACL antibody in any of the supernatants.

Modification of the incubation conditions to allow ACL antibody to bind to CL on the surface of the plate at 4°C, rather than 37°C also failed to detect ACL in the culture supernatants.

An increase in the concentration of CL to 40 g/ml still did not improve the sensitivity of the assay, in accordance with the findings of Radway-Bright (personal communication).

Discussion

It can be seen from the data presented that although it is possible, using the technique described, to measure IgG and IgM in supernatants of cultured PBMCs, it was not possible to detect spontaneous anticardiolipin antibody production in a reliable way.
This is in marked contrast to work in progress in this Department where a similar technique has been used for the detection of both double-stranded and single-stranded anti-DNA antibodies (Tyrell-Price, personal communication) and it has also been shown that antibody production can be manipulated by the addition of IL-10 and IL-12.

On reflection, it was perhaps optimistic to presume that it would be possible to detect anticardiolipin antibodies in culture. Total IgG and IgM were present in the supernatants at detectable levels but it should be borne in mind that these antibodies included both autoantibodies and those directed against extraneous antigens released owing to the generalised state of hyperactivation which exists in the immune system of SLE patients.

Anticardiolipin antibodies are probably produced by a very small number of the plasma cells. For the most part, plasma cells are located in the lymph nodes and not circulating in the peripheral blood so it could be viewed as unlikely that a plasma cell producing anticardiolipin antibodies would be isolated from a relatively small volume of peripheral blood. This hypothesis is rendered less likely when considering the fact that other autoantibodies are detectable in cell culture supernatants. We propose the possibility that while ACL-producing cells may be present in low numbers within the peripheral circulation, they may only occur in such small numbers as to be effectively undetectable. Another possibility is that phospholipids present in the culture supernatant derived from apoptotic cells could be cross-reacting with ACL antibody produced by cultured cells, thus neutralising the antibodies and rendering them undetectable in the ELISA system.

All of the patients sampled demonstrated significant levels of anticardiolipin antibodies in the plasma. However, given that the half life of serum immunoglobulin is around 21 days, this does not necessarily reflect the levels of antibody being produced currently. Free antibody circulating in the blood is likely to have been released into the circulation over the preceding weeks and might accumulate at higher levels than could be detected over a 6-day culture period, even assuming that anticardiolipin antibody producing cells were present in the culture.

The major problem in the development of an assay system such as this, supposing that detectable levels of the desired antibody were made, is that of determining precisely what is happening. PBMCs will all be producing cytokines which act in either an autocrine or a paracrine manner. The cytokine system demonstrates great redundancy and it is certainly possible that, should one be removed from the system by the addition of antibody, then another would be able to compensate. Also, cytokines are pleiotropic and the same cytokine may be capable of fulfilling different functions relative to the same cell depending on the ambient conditions.

Therefore, while the failure to develop this in vitro culture technique for the monitoring of the production of antibody, when a similar system was able to detect the production of anti-DNA antibodies, is disappointing as this method could have provided an initial measure of the possible effects of cytokines on spontaneous antibody production, given the numerous problems experienced there is little to be gained from further perseverance.

References